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[COMMUNICATION]

Estrogen Stimulates Peptidylarginine Deiminase Biosynthesis in the Primary Culture of Dispersed Rat Anterior Pituitary Cells

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ABSTRACT—Effects of sex steroids on peptidylarginine deiminase biosynthesis were examined in the primary culture of dispersed rat anterior pituitary cells. Without steroids, cells from 7 week to 6 month-old rats incorporated [^{35}S]-amino acids into the immunoprecipitable enzyme protein. No incorporation was detected in cells from 4 week-old rats. The cells responded to 10 nM 17β-estradiol (E_2) by an increase in the rate of enzyme biosynthesis regardless of sex and age of the rats. Diethylstilbestrol was as effective as E_2, whereas testosterone and progesterone had no effect. Dot blot hybridization analysis demonstrated an increase in the znzyme mRNA level in the E_2-stimulated cells. The results support that estrogen up-regulates the peptidylarginine deiminase expression in the rat pituitary.

INTRODUCTION

Peptidylarginine deiminase (protein-L-arginine iminohydrolase; EC 3.5.3.15) converts arginine residues in proteins to citrulline residues (reviewed in [1]). The enzymes reported thus far can be classified into 3 types based on the differences in their tissue distribution, antigenicity and substrate specificity [2]. Among them, the muscle type enzyme distributes in a widest variety of tissues which differ morphology and function, and has been best characterized biochemically [2–4]. It is present in lactotrophs of the mature female rat pituitary and its content varies under the influence of estrogen [5, 6], suggesting possible involvement of the enzyme in female specific activity of lactotrophs. We have previously reported that estrogen stimulates the enzyme biosynthesis in a clonal rat pituitary cell line [7]. However, such an established cell line may have different functional characteristics from normal pituitary cells. Therefore, the present study has been done using primary cultures of dispersed rat anterior pituitary cells. The results confirm the data obtained in our previous *in vitro* and *in vivo* studies [6, 7], suggesting that estrogen up-regulates the expression of peptidylarginine deiminase in the rat pituitary.

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MATERIALS AND METHODS

Wistar rats derived from Japan SLC (Shizuoka, Japan) were bred in the animal facility of the Tokyo Metropolitan Institute of Gerontology, and immature (4 weeks of age) to mature (4–6 months) males and females were used. Rats were decapitated under light ether anesthesia, pituitaries were removed aseptically, and the anterior lobes were isolated under a dissecting microscope. The cells were dispersed with trypsin-EDTA solution (GIBCO, Grand Island, NY) according to the previously described method [8]. After washing with the medium, the cells from 6–10 lobes were resuspended at $4–5\times10^5$ cells/ml, and dispensed at 3

ml/well into 6 well plates (Corning Glass Works, Corning, NY). The culture medium was a phenol red-free DMEM/F-12 mixture (Sigma, St. Louis, Mo.) supplemented with 10% normal horse serum, 2.5% fetal bovine serum, 2.85 g/l of $NaHCO_3$ and 1.5 g/l of glucose. Sera were pretreated with dextran-coated charcoal to deplete free steroids [7]. After incubation for 4 days in 5% CO_2 and 95% air at 37°C, the cells were treated for 24 hr with steroids as described previously [7].

Cells were biosynthetically labeled with [^{35}S]-amino acids as described previously [7]. Briefly, monolayered cells in each well were incubated for 1 hr with 3.7 MBq Tran ^{35}S-Label (ICN, Costa Mesa, Ca.) in 1 ml methionine-free DMEM/F-12 medium. After incubation, the cells were lysed in 1% NP-40, 1 mM PMSF, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2, and the lysates were centrifuged for 20 min at $10000 \times g$. The supernatants were normalized for radioactivity incorporated into TCA-precipitable proteins and then immunoprecipitated with rabbit antisera against rat muscle peptidylarginine deiminase, growth hormone (GH) or prolactin (PRL) and protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography [7].

Total RNA fractions from cultured cells were prepared by the guanidinium/CsCl method and analyzed by dot blot hybridization technique as described previously [9]. Probes used for hybridization were peptidylarginine deiminasse cDNA [4] and chicken actin cDNA (Oncor, Gaithersburg, Md.) which were labeled using [^{32}P]-dCTP (New England Nuclear, Boston, Ma.) and the random priming DNA labeling kit (Boehringer, Mannheim, Germany).

RESULTS AND DISCUSSION

First, anterior pituitary cells from 6 month-old females were cultured without steroid and labeled with [^{35}S]-amino acids. Figure 1 shows a fluorograph of immunoprecipitates prepared from the cell extract, demonstrating incorporation of labeled amino acids into peptidylarginine deiminase (75 kilodalton (kd)), GH (22 kd) and PRL

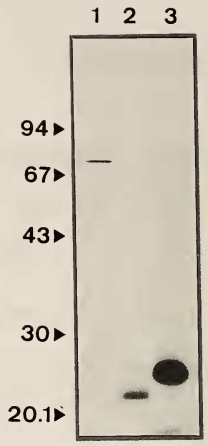

FIG. 1. Immunoprecipitation of [^{35}S]-labeled peptidylarginine deiminase, GH and PRL. Anterior pituitary cells from 6-month old females cultured without added steroid were labeled with [^{35}S]-amino acids. Peptidylarginine deiminase (lane 1), GH (lane 2) and PRL (lane 3) in the cell extract were immunoprecipitated and those from about 2×10^5 cells (lane 1) or 1×10^4 cells (lanes 2 and 3) were analyzed by SDS-PAGE. Molecular weight of marker proteins were given as kd.

(24 kd). When cells were incubated with 10 nM E_2 or DES, incorporation of labeled amino acids into the enzyme and RRL increased as compared with the control culture (Fig. 2). Testosterone had no appreciable effect, and progesterone seemed slightly suppressive on the incorporation into both the enzyme and PRL. None of these steroids affected the incorporation into GH. The observed effects of steroids on PRL biosynthesis are consistent with those reported previously [10].

Effects of E_2 on the peptidylarginine deiminase biosynthesis were examined similarly in cells from female and male rats at various ages (Fig. 3). Without E_2, no enzyme biosynthesis could be detected in cells from 4 week-old males and females. However, the enzyme biosynthesis was detectable in these cells upon E_2 stimulation. The enzyme biosynthesis was detectable in cells from 7 week- and 4 month-old rats of both sexes, although its activity was slightly lower in male cells than in female cells. The E_2 treatment increased the enzyme biosynthesis in these cells. It caused 5 to 10-fold increase in the rate of enzyme biosynthesis, as estimated by liquid scintillation counting of the

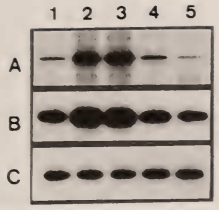

FIG. 2. Effects of various steroids on biosynthesis of peptidylarginine deiminase (A), PRL (B) and GH (C). Anterior pituitary cells from 6-month old female rats were preincubated for 24 h in the medium alone (lane 1) or with 10 nM of E_2 (lane 2), DES (lane 3), testosterone (lane 4) or progesterone (Lane 5), were labeled with [^{35}S]-amino acids, and the immunoprocipitates were analyzed by SDS-PAGE.

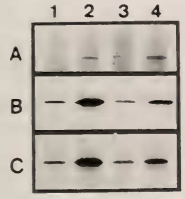

FIG. 3. Effects of E_2 on peptidylarginine deiminase biosynthesis in anterior pituitary cells from male and females rats at various ages. Cells were obtained from females (lanes 1 and 2) or males (lanes 3 and 4) at 4 weeks (A), 7 weeks (B) and 4 months (C) of ages and incubated for 24 hr with (lanes 2 and 4) or without (lane 1 and 3) 10 nM E_2. The cells were labeled and peptidylarginine deiminase was immunoprecipitated for SDS-PAGE analysis.

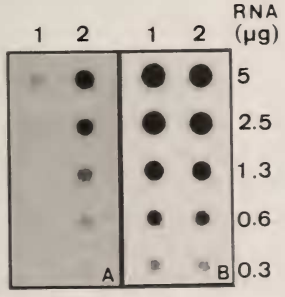

FIG. 4. Effects of E_2 on the peptidylarginine deiminase mRNA level of anterior pituitary cells. Cells from 5 to 6 month-old females were incubated for 24 hr with (lane 2) or without (lane 1) 10 nM E_2, and the total RNA fractions were analyzed by dot blot hybridization. Blots were first hybridized with a [^{32}P]-labeled peptidylarginine deiminase cDNA (A), and then the probe was stripped by immersing for 3 min in boiling water for reprobing with a labeled chicken actin cDNA (B).

immunoprecipitates (data not shown).

To see the effect of estrogen on the peptidylarginine deiminase mRNA level, anterior pituitary cells from 5 to 6 month-old females were cultured for 24 hr with or without 10 nM E_2 and the total RNA fractions were analyzed by dot blot hybridization. Figure 4 shows about 4 to 8-fold increase in the enzyme mRNA level by E_2 treatment. However, no detectable change was found in the actin mRNA levels.

These results agree with our previous data indicating that estrogen stimulates peptidylarginine deiminase biosynthesis in a clonal pituitary cell line [7]. It has been well established that the plasma estrogen level varies during the estrous cycle of the female rat [11]. Therefore, variation of the enzyme contents of the pituitary [6] and the uterus [5] during the estrous cycle may be, at least in part, brought about by the direct action of estrogen on these tissues. From the present and our previous studies [7] the stimulatory action of estrogen on the enzyme synthesis may be most likely mediated by increased transcription in a subset of cells. However, it may be also mediated in part by selective proliferation of a heterogeneous pituitary cell population.

Our previous study have shown that both male and female rat pituitaries contain barely detectable amounts of peptidylarginine deiminase at 3 weeks of age [6]. The enzyme content of female pituitaries markedly increases by 3 months showing an estrous cycle-related variation, whereas it remains low in males. The present findings on the enzyme biosynthesis in E_2-untreated cells are consistent with this ontogenic appearance of the enzyme in the female pituitary. However, cells from 7 week- and 4 month-old rats were found to synthesize substantial amounts of the enzyme regardless of sex. This apparent difference suggests some male-specific control may be operating *in vivo* to suppress the enzyme expression by pituitary cells.

Watanabe *et al.* [9] have recently reported an interesting variation pattern of the peptidylarginine deiminase mRNA level in female rat pituitaries around the estrous cycle. The mRNA level starts increasing at metesterus attaining the maximum level at diestrus, while the enzyme content attains its maximum level between proestrus and

estrus [6]. This shows, a sharp contrast to the *in vitro* findings that the estrogen-mediated increase in the enzyme content follows the mRNA increase with a short (less than 3 hr) time lag [7]. In addition, the magnitude of the mRNA variation is greater than 50-fold in the pituitary in contrast to only 4 to 8-fold increase observed *in vitro*. Therefore, additional factors may be involved in the regulation of peptidylarginine deiminase expression in the pituitary, which would modify the estrogen action at the levels of transcription, translation as well as mRNA stability.

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